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## INTRODUCTION

AIB1 (Amplified in Breast Cancer 1) is a member of the p160 family of steroid receptor coactivators. AIB1 is located on chromosome 20q and its gene is amplified in 5-10% of primary breast cancers (1). AIB1 mRNA is overexpressed in 60% of breast tumors (2) and its increased protein levels have been correlated with tumor size, p53 status and HER2/neu expression (3). High expression of AIB1 in breast tumors with high HER2/neu expression in patients that received tamoxifen therapy had the worst disease free outcome compared to all other patients (4, 5). AIB1 has been increasingly associated with function and regulation by growth factor non-steroid hormone induced signaling. Disruption of p/CIP, the mouse homolog of AIB1, results in a pleiotrophic phenotype including reduced female reproductive function and blunted mammary gland development in mice (6, 7) AIB1 has also been demonstrated to be a down stream target for phosphorylation by MAP kinase (8), IkappaB kinase (9) and is involved in regulating AKT kinase expression levels (10). These data reinforce the role of AIB1 in wide-ranging effects on tumor growth that is independent of steroid receptor function.

## BODY

The research accomplishments in this report include more data to address **Task 1** in the approved **Statement of Work** in the original grant application, DAMD17-02-1-0394. To continue to address the main goal of **Task 1**, examining the ability of AIB1 and Δexon3 AIB1 isoform to potentiate EGF and IGF-1 signaling in contributing to hormone independence, growth assays were utilized to measure the necessity of AIB1 and Δexon3 AIB1 isoform in IGF-1 and EGF growth signaling pathways. Anchorage independent growth is a critical hallmark of tumorigenesis and is therefore a particularly relevant measure of the importance of a gene's involvement in cancer. The

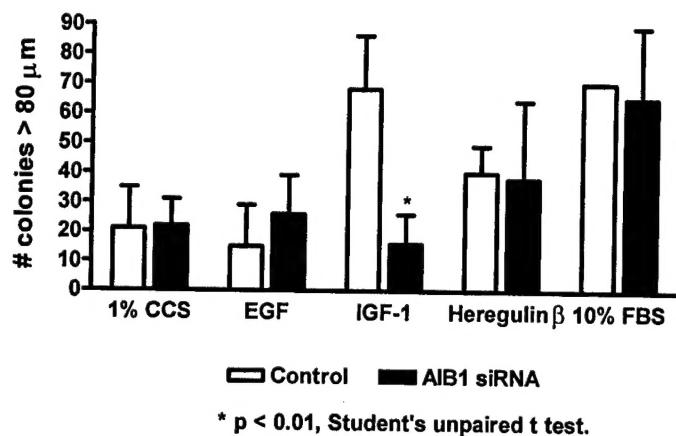
ability of AIB1 and Δexon3 AIB1 isoform to maintain anchorage independent growth of MCF-7 and MB-MDA-231 (MDA-231) cells was measured in the presence of IGF-1 or EGF and in the absence of hormones, such as estrogen. MDA-231 breast cancer cell line expresses EGF and IGF-1 receptors, but is estrogen receptor alpha negative. To modulate endogenous message levels of AIB1 and Δexon3 AIB1 isoform, a siRNA or small interfering RNA was designed against exon 7 to target the degradation of message and protein levels of AIB1 and Δexon3 AIB1 isoform (Figure 1). In AIB1 siRNA transfected cells, MCF-7 cells have a severely impaired ability to grow in the presence of IGF-1. EGF did not appear to have much of an effect on stimulating soft agar colony growth, whereas 10% fetal bovine serum seemed to rescue the MCF-7 cells from the loss of AIB1. MDA-231 cells did not respond has well to IGF-1 as MCF-7 cells to soft agar colony growth, however, the AIB1 siRNA transfected cells did have a severely limited ability the grow in 10% fetal bovine serum. In MCF-7 cells, AIB1 and Δexon3 AIB1 isoform have specific role in IGF-1 growth signaling whereas MDA-231 may have a more severe requirement for AIB1 and Δexon3 AIB1 isoform for maintaining anchorage independent growth.

The second part of **Task 1** in the approved **Statement of Work** was to analyze the mechanisms of the Δexon3 AIB1 isoform versus full length AIB1 potentiation in breast cancer. AIB1 was been shown to potentiate the promoter and message levels of the endogenous gene, cyclin D1, in response to estrogen (11). Cyclin D1 increases in expression in response to IGF-1 and EGF stimulation. Cyclin D1 is an important cell cycle regulating protein that is necessary for entry past G1 phases of the cell cycle. Changes in cyclin D1 expression could explain some of the growth changes observed in AIB1 and Δexon 3 AIB1 siRNA treated cells. To address the second part of **Task 1**, I wanted to determine if Δexon 3 AIB1 could further stimulate cyclin D1 message in the presence of IGF-1 as measured by quantitative real time PCR. AIB1 and Δexon 3 AIB1 expression

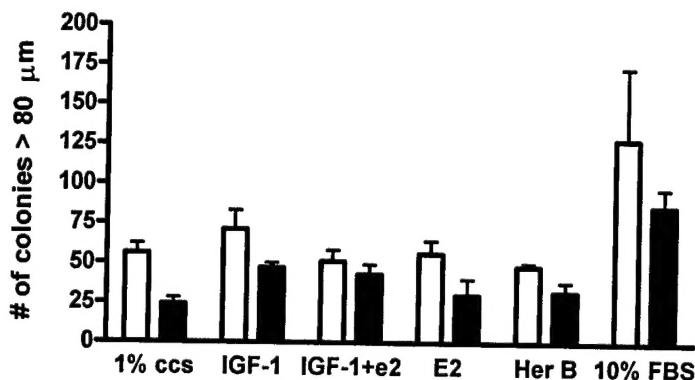
plasmids were transiently transfected into MCF-7 cells for 24 hours and then treated with estrogen or IGF-1 for 2 hours. I did not observe any further significant increases in cyclin D1 message when more AIB1 or Δexon 3 AIB1 were added to MCF-7 cells [Figure 2]. I did observe a slight increase in cyclin D1 message in response to estrogen with the addition of Δexon 3 AIB1 compared with control and AIB1 transfected. MCF-7 cells contain a gene amplification for the *aib1* gene and results in the expression of large amounts of AIB1 protein compared to other breast cancer cell lines. The addition of more AIB1 or Δexon 3 AIB1 by transient transfection may not result in additive effects as seen in Figure 2. Therefore, we used AIB1 and Δexon 3 AIB1 directed siRNA to lower the total amounts of protein and then observe any differences in the expression of Cyclin D1 protein in response to IGF-1 stimulation. MCF-7 cells were transfected with control or AIB1 siRNA and treated with or without IGF-1 for 48 hours. Whole cell lysates were harvested and lysates were run on a SDS-PAGE to resolve proteins according to size. The resolved proteins were transferred onto a PVDF membrane and a western blot analysis was done to examine specific levels of AIB1/Δexon 3 AIB1 and Cyclin D1 protein levels. The AIB1 western blot [Figure 3] demonstrates that the siRNA was able to effectively reduce protein levels of AIB1 and Δexon 3 AIB1. Cyclin D1 protein levels in both the presence and absence of IGF-1 are decreased by 30-40% in AIB1 siRNA transfected cells compared to the control. The beta actin western blot was used as a loading control. Expression of AIB1 and Δexon 3 AIB1 are critical for maintaining the expression of Cyclin D1 and potentiating its expression in the presence of IGF-1.

# Figure 1

A.

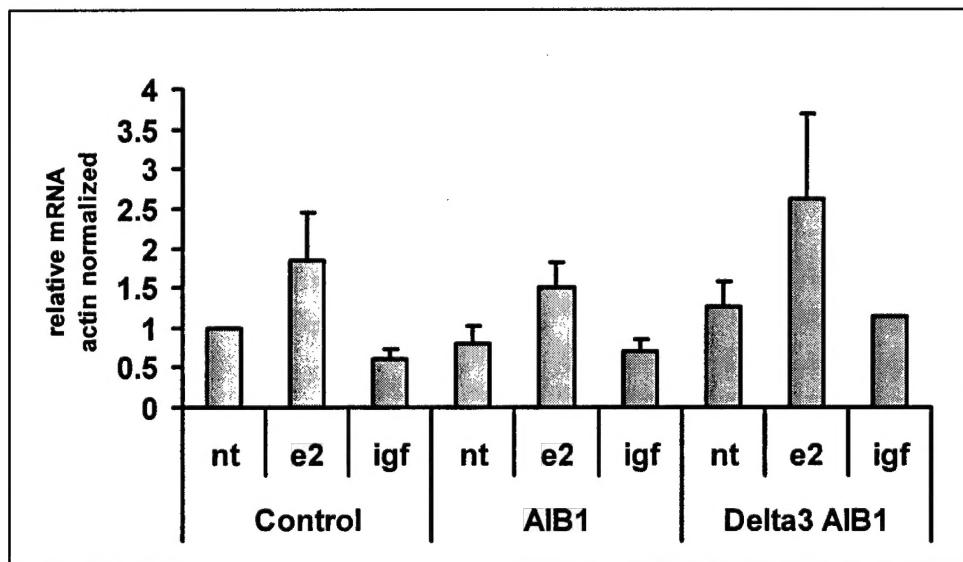


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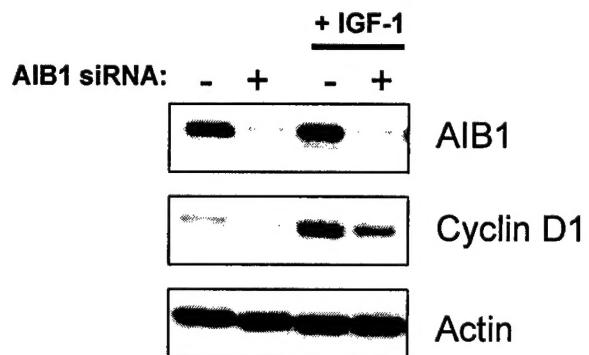
**Figure 1a and 1b. AIB1 and delta exon3 have effects on anchorage independent growth of breast cancer cells.** A pool of MCF-7 or MDA-231 were transfected with either control or AIB1/ delta exon3AIB1 siRNA for 24 hours and replated in 0.35% soft agar in the presence or absence of growth factors. 10% fetal bovine serum was used as a positive control. Soft agar colonies were measured after 2 weeks. Experiments were done in triplicate. White bars represent the control siRNA transfected samples. Control siRNA is a scrambled non-specific sequence that does not target any known mammalian genes. Black bars represent the AIB1 siRNA transfected cells

## Figure 2



**Figure 2. Transiently Transfecting AIB1 or  $\Delta$ exon3 AIB1 isoform into MCF-7 cells did not result in the increase mRNA levels of the endogenous gene cyclin D1.** MCF-7 cells were transfected with expression plasmids for AIB1 or delta exon 3 AIB1 for 24 hours. The transfected cells were treated with estrogen ( $10^{-8}$  M) or IGF-1 100 ng/ml for 2 hours. Total RNA was harvested and analyzed by reverse transcriptase real time pcr.

## Figure 3



**Figure 3. Reducing AIB1 and Δexon3 AIB1 isoform by siRNA transfection results in the reduction of cyclin D1.** Estrogen stripped MCF-7 cells were transfected with siRNA and treated with or without IGF-1 100 ng/ml for 48 hours. Whole cell extracts were harvested and protein levels were analyzed by western blot.

Status of Task 2: Experiments described in the approved grant application are still ongoing.

### KEY RESEARCH ACCOMPLISHMENTS

- Small interfering RNA (siRNA) directed against both AIB1 and Δexon 3 AIB1 message is an effective tool for reducing protein expression.
- AIB1 and Δexon 3 AIB1 is critical for IGF-1 stimulated anchorage independent growth of MCF-7 breast cells.
- MB-MDA-231 breast cancer cells have a different requirement for AIB1 and Δexon 3 AIB1 in anchorage independent growth.
- AIB1 and Δexon 3 AIB1 is necessary for maintaining the expression of Cyclin D1 and potentiating its expression in response to IGF-1.

### REPORTABLE OUTCOMES

Papers published:

Reiter, R., Oh, A.S., Wellstein, A., and Riegel, A.T. The Impact of the Nuclear Receptor Coactivator AIB1 isoform AIB1-Delta3 on Estrogenic Ligands with different intrinsic activity. Oncogene. 2004 Jan 15; 23(2): 403-9.

### CONCLUSIONS

The majority of experiments in Task 1 of the approved grant involving the co-activating effect of AIB1 and Δexon 3 AIB1 on EGF signaling in a breast cancer model has been completed and has begun to yield some positive results. It is clear from the experiments measuring anchorage independent growth in MCF-7 cells that AIB1 and Δexon 3 AIB1 has a major role in IGF-1

stimulated growth and potentiating the expression of IGF-1 responsive genes such as cyclin D1. It is of high interest to determine other genes that are involved in anti-apoptosis and cell cycle progression that are involved in maintaining growth in anchorage independent conditions and require AIB1 and Δexon 3 AIB1 for its expression.

ABBREVIATIONS:

IGF-1 – insulin-like growth factor 1  
EGF – epidermal growth factor  
MAP kinase – mitogen activated protein kinase  
Akt kinase – Protein kinase B  
siRNA – small interfering RNA

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